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(54) Title: POLYNUCLEOTIDE PROBE, METHOD AND KIT FOR THE IDENTIFICATION AND DETECTION OF GRAM-NEGATIVE BACTERIA

(57) Abstract

A polynucleotide probe (DNA or RNA) is provided, which probe recognizes a genus or species of Gram-negative bacteria, in particular an Enterobacteriaceae genus or species. The probe corresponds to a relatively short fragment of an outer membrane protein of the bacterium and is highly specific (exclusive and inclusive) for the genus or species under investigation. The probe or a combination of probes directed to different genera or species is used in a method for monitoring bacterial contamination, e.g. in foodstuffs or biological fluids, and can be provided as a diagnostic kit.

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Polynucleotide probe, method and kit for the identification and detection of Gram-negative bacteria

The invention relates to a polynucleotide probe which specifically recognizes a genus or species of Gram-negative bacteria.

The rapid detection of microorganisms is of great importance in a wide variety of sectors. In the foodstuffs sector, a rapid detection is of great economic significance because it enables a conclusion to be drawn with regard to the microbiological quality of raw materials, intermediates and end products. By this means the more rapid release of products for consumption is promoted and expensive and quality-lowering storage and waiting procedures are avoided. The fact that, for example, it takes at least 5 days to detect a Salmonella species in foodstuffs using the current microbiological methods can serve as an illustration of the need for accelerated microbiological determination.

Many genera of bacteria which play a significant role in foodstuffs microbiology belong to the family of the Enterobacteriaceae. Thus, the genera Salmonella, Shigella and Yersinia are pathogenic and involved to a large degree in recorded foodstuff infections. The specific detection of pathogenic Enterobacteriaceae is in many cases not only a time-consuming matter but, above all, difficult, especially when these bacteria belong to the minority flora, and this is usually the case. In foodstuffs microbiology this problem is frequently circumvented by using indicator organisms to determine the microbiological quality or carry out preventive checks. Thus, the presence of Enterobacteriaceae, without specification, in a foodstuff is used as an indication of the state of hygiene in foodstuffs companies. Determinations of this type are regularly carried out both by government agencies and monitoring bodies and by industry laboratories. Although the use of indicator organisms, as mentioned above, has proved its worth, it is nevertheless frequently necessary to carry out an identification for diverse species.

A rapid determination of microorganisms is of great impor-

tance in medicine and health care also, for example for localizing and combating infections. Furthermore, there is also a need for rapid and effective methods of determination for the presence of bacteria in the environmental sector.

In order to detect bacteria it has usually been necessary, up to now, to prepare a pure culture from the sample to be tested, after which conclusions are drawn on a specific species or a specific group on the basis of the form and other investigated properties of the bacteria. Thus, according to the widely used so-called API system, the microorganism is subjected to a large series of tests in each of which it is determined whether or not a specific enzyme activity occurs. The combination of activities found gives an indication for the species of bacteria or for the genus of bacteria in question. Methods of this type are laborious and, above all, not definitive: a bacterium or group of bacteria is detected only with a specific probability (of less than 100%).

Work is being carried out on various routes to obtain more rapid diagnostic methods for detecting bacteria. DNA-DNA hybridization is regarded as a highly promising technique (see Klausner and Wilson, Bio-Technology 1, 471-478, 1983). In principle, with this method a DNA fragment which is specific for a species of bacteria or a group of bacteria is sought as a "probe" for structurally related DNA in bacteria which it is desired to identify. The great advantage of a detection technique of this type is that testing is for the genotype instead of, as customary, for the phenotype. Characteristics which do not become expressed, or do so only with difficulty, and frequently can be tested only via animal experiments (for example kerato-conjunctivitis test for enteroinvasive E. coli and Shigella) can be tested in vitro via DNA-DNA hybridization. Moreover, the sensitivity of the technique is such that a number of preliminary treatments to which the material must be subjected in the conventional technique are not necessary. A review of the use of DNA probes is to be found in J.A. Matthews and L.J. Kricka, Analytical Strategies for the use of DNA probes: Anal. Biochem. 169, 1-25 (1988).

The route which has been followed up to now for developing DNA probes for bacteria is the fragmentation of bacterial DNA in a

relatively arbitrary manner, cloning fragments in plasmid vectors and investigating, with the aid of radioactive markers, whether fragments hybridize specifically with bacterial DNA. In order to use the highly promising PCR procedure, the sequence of such fragments must then be determined, after which oligonucleotides are prepared and tested again. The disadvantage of probes of this type is that they demand a prolonged and laborious preparation, a preparation which, moreover, must be developed entirely anew for a subsequent species of bacteria to be investigated, as a result of which the composition of a kit for determining various bacteria in samples on the basis of such probes is virtually impossible. Probes based on ribosomal RNA are also known: however, in a number of cases these display a troublesome cross-reactivity (inadequate inclusivity and exclusivity).

It has been found that certain sequences of genes which code for outer membrane proteins of Gram-negative bacteria and the sequences complementary hereto are very suitable as a polynucleotide probe with which microorganisms of a specific species or genus can be detected, for example in foodstuffs, in a clinical specimen or in the environment.

The polynucleotide probe according to the invention therefore contains a polynucleotide which corresponds with or is complementary to a fragment of a gene or messenger RNA coding for an outer membrane protein of a Gram-negative bacterium.

An advantage of the polynucleotide probe according to the invention is that this contains only a relatively small number of nucleotides (of the order of 10-40 base pairs), as a result of which this can be simply prepared and used. At the same time, this probe proves to be specific for a certain genus or species of Gram-negative bacteria.

The polynucleotide probe can contain either a polydeoxyribonucleotide (DNA probe) or a polyribonucleotide (RNA probe). In this context the term "polynucleotide" does not indicate a specific minimum number of nucleic acids per molecule and this term is therefore not in absolute contrast to "oligonucleotide".

A further advantage of the polynucleotide probe according to the invention is that the hybridization can also be made detectable

by other methods, for example by the so-called PCR (polymerase chain reaction) method. Furthermore, the probes are relatively easy to combine into a kit with which a series of relevant Gram-negative bacteria can be identified, for example in foodstuffs, body fluid, water samples and the like.

Outer membrane proteins are proteins which are present in the outer membrane of the cell envelope of Gram-negative bacteria, where they fulfil various functions such as allowing the passage of nutrients (the pore-forming outer membrane proteins or porines) and of larger dissolved substances and contributing to the structure and the binding of the outer membrane. These proteins are designated OmpA, OmpC, OmpF, PhoE, LamB, Tsx, OmpT, FhuA, BtuB, FepA, FecA, etc.

Outer membrane proteins of E. coli are described by J. Tommassen in Membrane Biogenesis by J.A.F. Op den Kamp, NATO ASI Series, Vol. H16, 351-373 (1988), Springer Verlag, Berlin-Heidelberg. The amino acid sequence of outer membrane protein F of Pseudomonas aeruginosa and the corresponding DNA sequence are described in EP-A-297.291

The outer membrane proteins of Gram-negative bacteria contain fragments which are highly heterologous, that is to say significant differences in the amino acid sequence of the fragments of the proteins exist between the various microorganisms. As a result of this pronounced heterology, a differentiation between species or genera of bacteria can be made with relatively short sequences. Consequently, the genes coding for these proteins also locally display a pronounced heterology. These genes are correspondingly designated ompA, ompC, ompF, phoE, etc. The phoE genes of a few enterobacteria have been investigated by Van der Ley et al., Eur. J. Biochem. 164, 469-475 (1987). The ompA genes of a few enterobacteria have been described by Braun & Cole, 1984, Mol. Gen. Genet. 195, 321-328.

The heterologous fragments of the outer membrane proteins frequently occur in the parts of the outer membrane proteins which are exposed at the cell surface, while the membrane-bridging parts are substantially preserved. Therefore, the fragments of the genes which correspond to the exposed fragments of the outer membrane

proteins are particularly suitable as a basis for specific polynucleotide probes according to the invention.

An example of a suitable probe for detecting Salmonella species is a probe which contains the 23-mer oligodeoxynucleotide TTTAGTAGACGGGCCGCCAGGGA, corresponding to an exposed part of the OmpA protein of S. typhimurium.

The probes according to the invention can be built up on the basis of the outer membrane proteins, in principle of all Gram-negative bacteria, and therefore are used for detecting the same Gram-negative species or genera of bacteria. Probes for Enterobacteriaceae are particularly important because these bacteria play a major role in infections, food poisoning, biological contamination and the like. Bacteria of the genera Enterobacter, Klebsiella, Salmonella, Yersinia, Edwardsiella, Erwinia, Serratia, Citrobacter, Proteus and Providencia may be mentioned. The invention can also be used for detecting Gram-negative bacteria other than Enterobacteriaceae, such as Pseudomonas.

The polynucleotide probes according to the invention recognize specific genera of bacteria and possibly species of bacteria. Thus, a probe derived from an outer membrane protein of Salmonella typhimurium proves to recognize all Salmonella species and not to show any false-positive reaction with other species. In this context, however, it should be stated that the current categorization of bacteria in genera and species is by no means perfect, as a result of which it can arise that a probe derived from a species or strain belonging to a specific genus does not recognize a species with the same genus name.

However, the genus restriction of the probes according to the invention is so pronounced that the occurrence of such a "false-negative" reaction is an indication that the species which gives this negative reaction does not belong to the genus in question. Conversely, a probe derived, for example, from an Escherichia coli also recognizes Shigella sp., which confirms that E. coli and Shigella sp. belong to the same genus and perhaps even to a single species (see W.J. Brenner in Bergey's Manual of Systematic Bacteriology, vol. I, pp. 410-411, Williams & Williams Co. Baltimore (1984)).

The fact that the polynucleotide probes according to the invention have such a high exclusivity and inclusivity is particularly surprising in view of the results with DNA probes according to the prior art.

The sensitivity (inclusiveness) of the probes as also surprising beacuse of the heterogeneity obeserved in the outer membrane protein pattern of different isolates of a single species. For instance, Overbeeke and Lugtenberg (J. Gen. Microbiol. 121 373-380 (1980)) compared the outer membrane protein patterns of 45 different E.coli isolates by SDS-PAGE and observed 36 different types among these strains. Therefore, the sensitivity of the probes based upon the variable parts of outer membrane was not to be expected.

The polynucleotide probe according to the invention can be prepared on the basis of a suitable fragment of a gene or messenger RNA coding for an outer membrane protein of the chosen bacterium by lining up together the relevant nucleotides according to a procedure known per se. The preparation can advantageously be automated using a DNA synthesis installation.

The ability of the probe thus obtained selectively to detect bacteria is demonstrated, for example, by means of hybridization in a so-called slot-blot procedure (P.J. Carter et al., Cell 38, 835-840 (1984)). In this procedure the DNA of the bacterium or bacteria to be investigated is liberated from the cells and attached to a filter and then brought into contact with a probe according to the invention, provided with, for example, a radioactive label. After hybridization it is investigated, for example by means of an autoradiogram, whether the sample has reacted with a nucleotide sequence, in other words has recognized this nucleotide sequence.

On the other hand, the so-called polymerase chain reaction (PCR) procedure, see R.K. Saiki et al., Science 239, 487 (1988) and EP-A-200.362, can advantageously be used for the detection of hybridization. With this procedure, a polydeoxynucleotide fragment is efficiently enzymatically reproduced in copying and denaturing cycles at different temperatures with the aid of two short oligodeoxynucleotides, so-called primers, occurring in a long polydeoxynucleotide. In this way, such a large amount of a recognized DNA

fragment can be obtained with two probes in a short time that detection without radioactive labelling is possible.

Another recently developed procedure which can be used to detect hybridization is the coupling to viral RNA, after which the probe is hybridized with the DNA sample to be determined. Any hybridization can then be demonstrated by in vitro reproduction of the hybridized RNA with the aid of viral replicas. In this way such a large amount of the hybridized RNA can be obtained in a short time that detection without radioactive labelling is possible. See also P.M. Lizardi et al., Biotechnology 6, 1197-1102 (1988).

The polynucleotide probe can be used selectively to detect a species or genus of bacteria within a multiplicity of bacteria, for example in foodstuffs. The polynucleotide probe according to the invention is also very suitable for identifying isolated bacteria, where appropriate in a pure culture, for medical use (infections).

The invention therefore also relates to a method for the detection and identification of Gram-negative bacteria, in particular of Enterobacteriaceae, using the polynucleotide probe as described above.

The method consists, for example, in isolating the polynucleotide material from the sample to be investigated, or attaching it to a carrier, in a known manner and then bringing it into contact with a probe according to the invention. The hybridization can then be detected by various methods, for example by using ^{32}P-ATP, viral replicas etc. The method according to the invention is particularly advantageous when using the PCR method as indicated above, in which case the polynucleotide fragment reproduced can be detected in various ways.

The invention also relates to a kit for the selective detection/identification of Gram-negative bacteria, which kit contains one or more of the polynucleotide probes described above. In particular, this kit contains a combination of DNA probes for testing for the presence of a series of Enterobacteriaceae.

In addition to the polynucleotide probe, the kit according to the invention can also contain means for isolating the (hybridized) polynucleotide, such as filters, for detecting the hybridization

signal, such as labelling substances, and for in vitro reproduction of hybridized polynucleotide fragments, such as a DNA-polymerase or a viral polymerase, and also other means for carrying out an identification of bacteria, and if desired a method for this.

The examples below illustrate that polynucleotide sequences which code for parts of outer membrane proteins exposed at the cell surface, or the sequences complementary to these, can be used as genus-specific probes for the identification of Gram-negative bacteria.

Example I

Synthesis and labelling of oligonucleotides

The oligodeoxynucleotides are prepared automatically with the aid of a Biosearch 8600 DNA synthesizer and are then purified by means of high pressure liquid chromatography. The oligomers are labelled at the 5'-end by enzymatically catalyzed transfer of ^{32}P phosphate from [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Int. plc, Amersham, England) with T4 polynucleotide kinase (Pharmacia, Uppsala, Sweden) using the method of Maniatis et al., Molecular Cloning, a laboratory manual, Cold Spring Harbor, N.Y. (1982).

Bacterial strains and plasmids

The following bacterial strains were cultured overnight at 37°C with shaking in L medium (J. Tommassen et al., EMBO. J. 2, 1275-1279 (1983)). Escherichia coli K-12 strain CE1194 with a chromosomal phoE deletion, and the phoE derivative CE1195 (J. Tommassen et al., J. Bacteriol. 149, 668-672 (1982)). E. coli B and C (Institute for Molecular Biology and Medical Biotechnology, University of Utrecht). E. coli strain S1, S2, S3, S4, S5, S6, S8 and S9, isolated from blood cultures of patients having bacteremia, F1, F5, F6, F9 and F12 isolated from faeces of healthy volunteers, and U6, U7, U8, U11 and U13 isolated from urine of patients having urinary tract infections, are described by Overbeeke and Lugtenberg (J. Gen. Microbiol. 121 373-380 (1980). Strain EIEC, an enteroinvasive E. coli from human faeces and Shigella dysenteriae, Shigella boydii serovar 2 and an unknown serovar, Shigella flexneri serovars I, II, III, IV, VI, X and an unknown serovar, Shigella sonnei serovars I, II and an unknown serovar from human faeces were obtained from the State Institute of Health and Environmental

Hygiene, Bilthoven. Other enterobacterial strains used were Edwardsiella tarda, Enterobacter aerogenes, Citrobacter freundii, Klebsiella pneumoniae, Proteus mirabilis, Providencia stuartii, Salmonella braenderup, Salmonella derby, Salmonella panama, Serratia marcescens, Shigella flexneri, Shigella sonnei (H. Hofstra, J. Dankert, J. Gen. Microbiol. 119, 123-131 (1980), Salmonella typhimurium 8J2353 (T. Sato, T. Yura, J. Bacteriol. 139, 468-477 (1979)), Salmonella typhimurium LT_2, Salmonella typhimurium B7121-2 pro$^-$ (Institute for Molecular Biology and Medical Biotechnology, University of Utrecht) and Salmonella typhimurium KB1711 (K. Bauer et al., J. Bacterol. 161: 813-816 (1985)). The following were used for the polymerase chain reaction procedure: E. coli K-12 strain CGSC4234 (E. coli Genetic Stock Center, Department of Human Genetics, Yale University School of Medicine, New Haven, Conn.), Salmonella panama and Salmonella typhimurium SJ2353. The following plasmids were used for the DNA spot tests: pACYC184 (A. Chang, S. Cohen, J. Bacteriol. 134: 1141-1156 (1978)) and its derivatives pJP29 (contains the phoE gene of E. coli, D. Bosch et al., J. Mol. Biol. 189, 449-455 (1986), pKP_2 (contains the phoE gene of Klebsiella pneumoniae, P. van der Ley et al., Eur. J. Biochem. 164: 469-475 (1987) and pEC17 (contains the phoE gene of Enterobacter cloacae, C. Verhoef et al., Gene 32: 107-115 (1984), plasmid pBR322 (F. Bolivar et al., Gene 2: 95-113 (1977)) and its derivative pST1 (contains a portion of the phoE gene of Salmonella typhimurium, G. Spierings, not published).

Slot-blot hybridization procedure:

After the optical density at 600 nm of the cell cultures obtained had been adjusted to OD = 1.1 for testing of the Shigella probe and to OD = 0.7 for testing of the Salmonella probe, amounts of 100 µl were filtered slowly through nitrocellulose (NC) filters (BA 85-Schleicher and Shuell, Kassel, Federal Republic of Germany) in a slot-blot apparatus (Minifold II, Schleicher & Shuell). The filters were prepared using the method of P.J. Carter et al., Cell 38, 835-840 (1984). The DNA was attached to the NC filter by UV irradiation (≈ 320 nm, L2723, Ankersmit) for 4 minutes. The filters were pre-hybridized for 45 minutes at 60°C in 0.25% Protifar (Nutricia NV, Zoetermeer), 6 x SSC (90 mM sodium chloride, 90 mM

sodium citrate pH 7.0). After adding the probe (10 pmol DNA), the filter was hybridized for 1.5 hours at 60°C. The filters were washed twice with 6 x SSC at 60°C and autoradiograms of the filters were made by exposure to a Fuji X-ray film for 3 days.

DNA spot procedure: 1 µl of the plasmid solutions (≈ 0.5 µg/µl) was spotted on NC. The filters were then treated and hybridized as described in the slot-blot hybridization procedure.

Polymerase chain reaction (PCR) procedure: Taq DNA polymerase (Perkin Elmer CETUS, Norwalk, USA) was used in accordance with the manufacturer's instructions except for the use of 10 mM Tris . HCl, pH 8.8 in place of pH 8.4, 1 unit of Taq DNA polymerase in place of 2 units and the final volume was reduced from 50 µl to 25 µl. Chromosomal DNA (≈ 10 ng), isolated by the method of Marmur (Marmur, J. et al., J. Mol. Biol. 3, 208-218 (1961) from the E. coli K-12 strain CGSC4234, S. panama and S. typhimurium SJ 2353), was used as substrate in the reaction. ST1/ST3c and ST2/ST3c were used as primer couples, resulting in the reproduction of fragments of, respectively, 285 and 156 base pairs. 40 cycles of 1 minute at 92°C, 2 minutes at 42°C and 2 minutes at 65°C were run with the aid of a programmable incubator block (PHC-1, New Brunswick Scientific B.V., Soest, Holland). 10 µl of the reaction mixture was analysed on a 1.3% agarose gel.

Salmonella probes: Three Salmonella probes were synthesized, based on the first, the second and the third fragment of the OmpA protein of S. typhimurium exposed at the cell surface. The DNA sequences of these fragments are indicated as ST1, ST2 and ST3c respectively in Figure 1. The inclusivity and exclusivity of ST3c was tested in a slot-blot hybridization procedure. Figure 4 shows the autoradiogram of the filter after hybridization with the probe. The following strains were applied to the filter: 1A, P. mirabilis; 1B, S. panama; 1C, S. typhimurium; 2A, S. marcescens; 2B, C. freundii, 2C, K. pneumoniae; 3A, E. tarda; 3B, E. aerogenes; 3C, P. stuartii; 4A, E. coli B; 4B, S. typhimurium KB1711; 4C, S. braenderup; 5A, S. derby; 5B, S. typhimurium LT2; 5C, S. typhimurium B pro; 6A, P. vulgaris; 6B, S. flexneri; 6C, S. sonnei. Streaks in the autoradiogram indicate that the probe was bound to a complementary nucleotide sequence in the lysed cells. The figure

shows that the probe reacts with S. braenderup, S. derby and S. typhimurium. The probe does not react with the other Enterobacteriaceae. However, under the test conditions used the probe does not react with S. panama. Under the conditions used in the PCR reaction (Figure 5), however, both the chromosomal DNA isolated from S. panama (Slots 5 and 6) and the chromosomal DNA isolated from S. typhimurium (Slots 3 and 4) were found to be used as substrate. When the chromosomal DNA isolated from E. coli was offered as substrate, amplification of the fragment of 285 base pairs (Slot 1) also took place when the probes ST1 and ST3c were used, and also of the fragment of 156 base pairs (Slot 2) when the probes ST2 and ST3c were used. The results show that the three probes are specific for Salmonella.

Example II

Shigella probe: For the development of the Shigella probe, use was made of the very close relationship which exists between Shigella and E. coli (see W.J. Brenner, Family I Enterobacteriaceae in: Bergey's Manual of Systematic Bacteriology, vol. I, The Williams & Wilkins Co., Baltimore, pp. 410-411 (1984)). It was therefore assumed that the sequences of the phoE genes of Shigella species are virtually identical to the known sequence of E. coli K-12 phoE gene. The Shigella probe for which the sequence is shown in Figure 2 together with the corresponding sequences of K. pneumoniae and E. cloacae (P. van der Ley et al., Eur. J. Biochem. 164: 469-475 (1987)) is based on the fifth portion of the PhoE protein of E. coli exposed at the cell surface. The specificity of the probe was tested with the aid of a slot-blot hybridization procedure. The autoradiogram is shown in Fig. 6. The following strains were applied to the filter: 1A, P. mirabilis; 1B, S. panama; 1C, S. typhimurium; 2A, S. marcescens; 2B, C. freundii; 2C, K. pneumoniae; 3A, E. tarda; 3B, E. aerogenes; 3C, P. stuartii; 4A, CE1195; 4B, CE1194; 4C, EIEC; 5A, Sh. sonnei I; 5B, Sh. sonnei II; 5C, Sh. sonnei; 6A, Sh. dysenteriae; 6B, Sh. boydii; 6C, Sh. flexneri; 7A, Sh. flexneri I; 7B, Sh. flexneri II; 7C, Sh. flexneri III; 8A, Sh. flexneri IV; 8B, Sh. flexneri X; 8C, L-medium; 9A, Sh. sonnei; 9B, Sh. boydii 2; 9C, Sh. flexneri VI. The probe reacted with all tested Shigella strains and with E. coli K12 CE1195 and the entero-

invasive E. coli strain (EIEC).

In a similar assay, the probe was tested on a number of E. coli strains isolated from blood cultures of patients having bacteremia, from faeces of healthy volunteers and from urine of patients having urinary tract infections. These strains represent a variety of O and K serotypes (as described by Overbeeke and Lugtenberg, J. Gen. Microbiol. 121 373-380 (1980)). Figure 7 shows that the probe reacted with all these different strains.

The results show that the probe is specific for the Shigella/E. coli species.

Example III

Enterobacter and Klebsiella probe:

The Enterobacter probe and the Klebsiella probe, the sequences of which are shown in Figure 3 together with the corresponding sequence of E. coli, are based on the eighth fragment of the PhoE protein of, respectively, E. cloacae and K. pneumoniae exposed at the cell surface. The specificity of the probes was investigated in DNA spot tests. The autoradiogram of the filter hybridized with the Enterobacter probe and hybridized with the Klebsiella probe is shown in Figure 8 on the left-hand photograph and right-hand photograph respectively. The following plasmids were applied to the filters: 1A, pEC17; 1B, pBR322; pJP29; 2B, pACYC184; 3A, pKP2; 3B, pST1. It can be seen on the left of Figure 8 that the Enterobacter probe reacts only with pEC17 and not with the other plasmids. It can be seen on the right of Figure 8 that the Klebsiella probe reacts only with pKP_2.

It can be concluded from this that the Enterobacter probe and the Klebsiella probe do not cross-react with the phoE genes of the other Enterobacteriaceae tested.

CLAIMS

1. Polynucleotide probe which specifically recognizes a genus or species of Gram-negative bacteria, which probe contains a polynucleotide which corresponds with or is complementary to a fragment of a gene or messenger RNA coding for an outer membrane protein of the Gram-negative bacterium.

2. Probe according to claim 1, characterized in that the fragment of the gene or messenger RNA codes for a portion of the outer membrane protein which is essentially exposed at the cell surface.

3. Probe according to claim 1 or 2, characterized in that the Gram-negative bacterium belongs to the Enterobacteriaceae.

4. Method for the detection and/or identification of Gram-negative bacteria, characterized in that one or more probes according to one of claims 1-3 is used.

5. Method according to claim 4, characterized in that Enterobacteriaceae are detected and/or identified using probes according to claim 3.

6. Method according to claim 4 or 5, characterized in that a fragment of the gene or messenger RNA which is recognized by the probes is then reproduced by using a polymerase chain reaction.

7. Kit for the detection and/or identification of Gram-negative bacteria which contains one or more polynucleotide probes according to claims 1-3.

8. Kit for the detection and/or identification of Enterobacteriaceae which contains one or more polynucleotide probes according to claim 3.

Fig-1

ST 1	TTCATTCACAATGATGGCCCGACT
ST 2	GACAACATCAATGGCGCTTATAAA
ST 3c	TTTAGTAGACGGGCCGCCAGGGA

Fig-2

K. PNEUMONIAE	TTTCGAACCCTGGCCGCGGGCCA
E. COLI	ACGCTTGCCTGTGCCACGGCTTT
E. CLOACAE	TTTCTGGCCCTGACCACGCGCCA

Fig-3

K. PNEUMONIAE	GATGTCGTCATCGTTGATGCCGAG
E. COLI	AATATCATCATTATTAATATTCAA
E. CLOACAE	AATATCATCGCTGCTTACGCCCAG

Fig-4

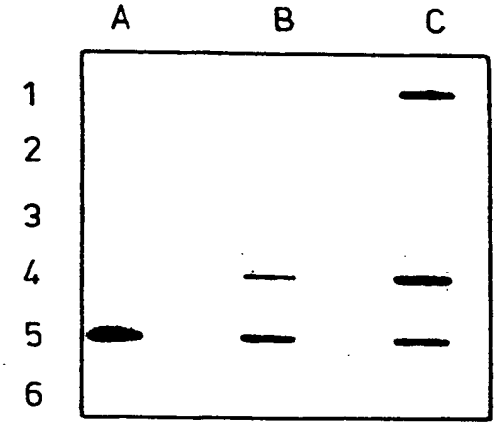


Fig-5

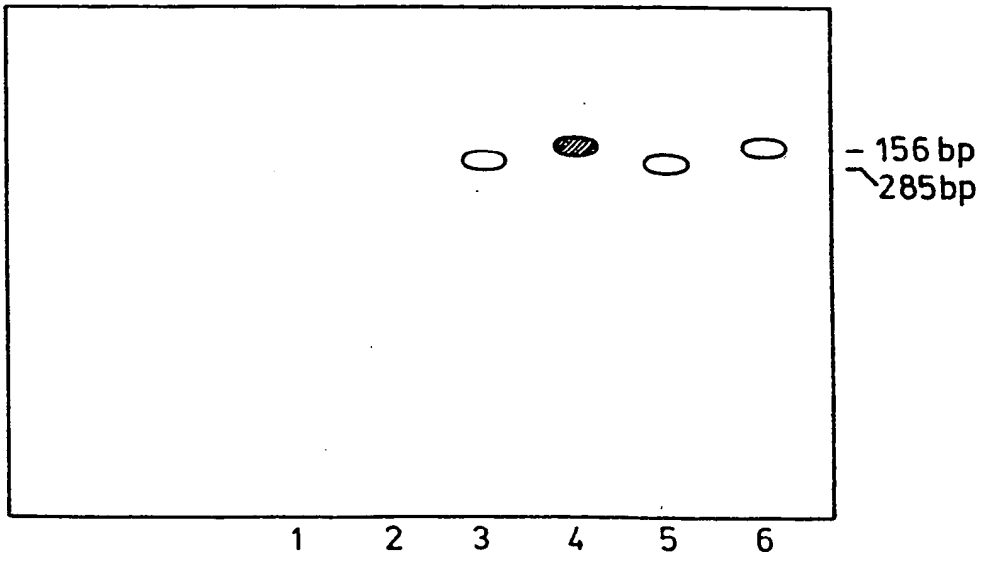


Fig - 6

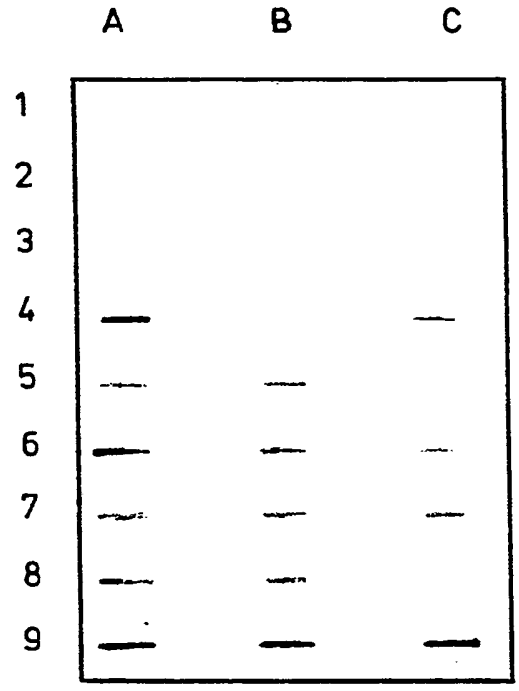


Fig - 8

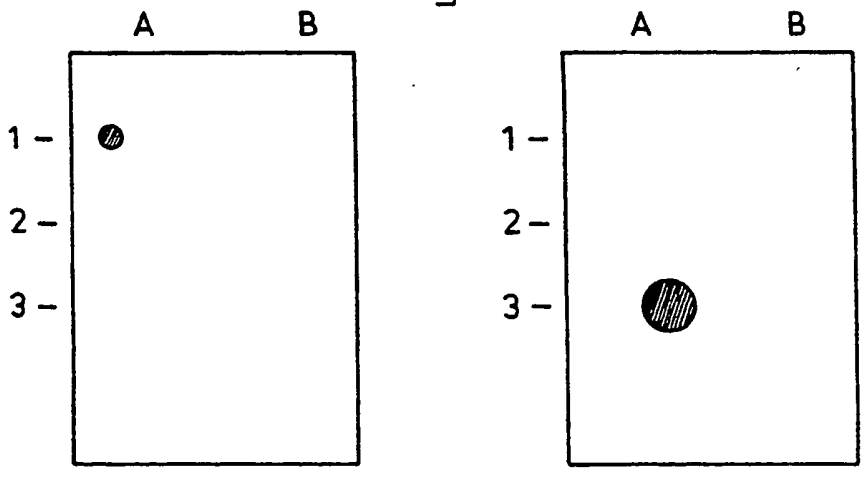
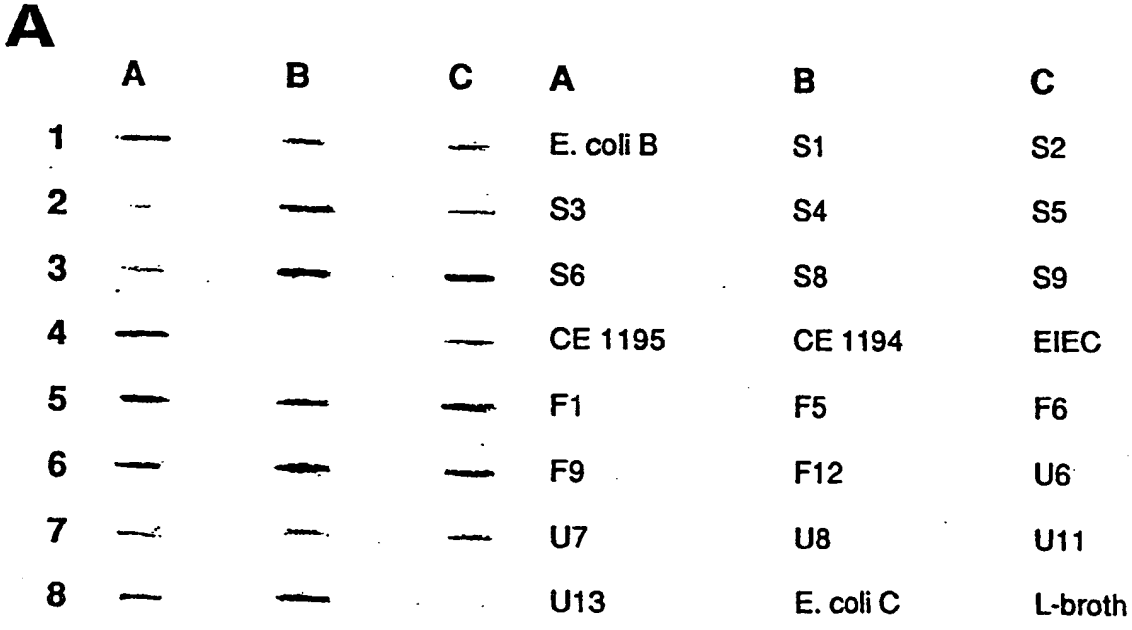


Fig-7


A
A
B
C
A
B
C
1
E. coli B
S1
S2
2
S3
S4
S5
3
S6
S8
S9
4
CE 1195
CE 1194
EIEC
5
F1
F5
F6
6
F9
F12
U6
7
U7
U8
U11
8
U13
E. coli C
L-broth

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 90/00033

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Journal of Clinical Microbiology, volume 18, no. 1, July 1983, American Society for Microbiology, (Washington, D.C., US), A.M. Palva: "OmpA gene in detection of Escherichia coli and other enterobacteriaceae by nucleic acid sandwich hybridization", pages 92-100 see the whole article	1-5,7,8
X	Journal of Bacteriology, volume 149, January 1982, (Washington D.C., US), S.T. Cole et al.: "Cloning and expression in Escherichia coli K-12 of the genes for major outer membrane protein ompA from Shigella dysenteriae, Enterobacter aerogenes, and Serratia marcescens", pages 145-150 see the whole article	1-5,7,8

./.

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21st June 1990	06 JUIL. 1990
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	PIRSS T. TAZELAAR

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
T	Applied and Environmental Microbiology, volume 55, no. 12, December 1989, American Society for Microbiology, (Washington, D.C., US), G. Spierings et al.: "Development of enterobacterium-specific oligonucleotide probes based on the surface-exposed regions of outer membrane proteins", pages 3250-3252 see the whole article	1-8
A	EP, A, 0297291 (BEHRINGWERKE AG) 4 January 1989 see column 4, example 2, page 9, table; column 11, lines 53-56	1-5,7,8

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. NL 9000033 SA 35550

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 29/06/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0297291	04-01-89	DE-A- 3718591	15-12-88
		AU-A- 1697788	08-12-88
		JP-A- 1085086	30-03-89